

Development of a Genome-Wide Random Mutagenesis System Using Proofreading-Deficient DNA Polymerase δ in the Methylophilic Yeast *Hansenula polymorpha*

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The thermotolerant methylotrophic yeast *Hansenula polymorpha* is attracting interest as a potential strain for the production of recombinant proteins and biofuels. However, only limited numbers of genome engineering tools are currently available for *H. polymorpha*. In the present study, we identified the *HpPOL3* gene encoding the catalytic subunit of DNA polymerase δ of *H. polymorpha* and mutated the sequence encoding conserved amino acid residues that are important for its proofreading 3'→5' exonuclease activity. The resulting *HpPOL3** gene encoding the error-prone proofreading-deficient DNA polymerase δ was cloned under a methanol oxidase promoter to construct the mutator plasmid pHIF8, which also contains additional elements for site-specific chromosomal integration, selection, and excision. In a *H. polymorpha* mutator strain chromosomally integrated with pHIF8, a *URA3^r* mutant resistant to 5-fluoroorotic acid was generated at a 50-fold higher frequency than in the wild-type strain, due to the dominant negative expression of *HpPOL3**. Moreover, after obtaining the desired mutant, the mutator allele was readily removed from the chromosome by homologous recombination to avoid the uncontrolled accumulation of additional mutations. Our mutator system, which depends on the accumulation of random mutations that are incorporated during DNA replication, will be useful to generate strains with mutant phenotypes, especially those related to unknown or multiple genes on the chromosome.

Key words: *Hansenula polymorpha*, random mutagenesis, DNA polymerase δ , mutator

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The non-conventional yeast *Hansenula polymorpha* is a thermotolerant methylotrophic yeast that has been regarded as an attractive model organism for basic research and biotechnological applications. *H. polymorpha* has been studied extensively for methanol metabolism, peroxisome biogenesis, nitrate assimilation, protein glycosylation, and various stress responses [9, 22, 42]. *H. polymorpha* has become one of the promising hosts for the production of recombinant proteins on an industrial scale owing to the availabilities of strong inducible promoters and a multi-copy integration system for target protein expression cassettes into the genome [6, 7, 10, 13, 15, 16]. In addition, this organism has several peculiar physiological characteristics, such as resistance to heavy metals and oxidative stress, and thermotolerance, which are advantageous for various biotechnological applications [4]. Furthermore, the capability of *H. polymorpha* for the alcoholic fermentation of glucose, xylose, cellobiose, starch, and xylan at elevated temperatures makes this organism an ideal candidate for lignocellulosic biomass-based ethanol fermentation. Recently, the whole genome sequence of *H. polymorpha* was determined [8, 31], and functional genomic approaches were developed to analyze the genome-wide responses to changes in metabolic or environmental conditions [26, 28, 43]. These advances will facilitate systems-level analyses and engineering. However, the currently available genome engineering tools for *H. polymorpha* are limited to those depending mainly on techniques based on homologous recombination, such as gene disruption based on Cre-loxP [30], gene targeting based on a non-homologous end-joining-deficient mutant [38], and random insertion techniques based on restriction enzyme-mediated integration (REMI) [21].

To obtain genetic variability in a microorganism by modifying proteins, metabolic pathways, or entire genomes so that they have the desired properties, various random mutagenesis techniques are used. Among them, chemical mutagenesis relying on the treatment of mutagens, such as ethyl methanesulfonate (EMS) and nitrosoguanidine (NTG), and radiation mutagenesis relying on exposure to different types of radiation, such as ultraviolet (UV) light and ionizing radiation, are the most widely used approaches owing to their relatively simple procedures and broad applicable organism ranges. However, these methods have a drawback for *in vivo* mutagenesis, as severe cell damage can be induced. A novel *in vivo* mutagenesis technique using error-prone proofreading-deficient DNA polymerase δ has been developed using the conventional yeast *Saccharomyces cerevisiae* [23]. It has been shown that mutant DNA polymerase δ with alteration of the residues responsible for 3'→5' exonuclease activity to remove mismatched nucleotides during DNA replication becomes proofreading-deficient and thereby functions as a strong mutator [25]. It has also been shown that the corresponding mutant DNA polymerase δ of *Schizosaccharomyces pombe* also functions as a mutator [3].

In this study, we identified the DNA polymerase δ of *H. polymorpha*, created a Pol3* allele encoding an error-prone proofreading-deficient DNA polymerase δ , and developed a mutator vector containing the *POL3** gene. We also developed and experimentally verified a strategy for genome-

wide random mutagenesis, which consists of a mutator strain created by the chromosomal integration of a mutator vector through targeted homologous recombination, Pol3*-based random mutagenesis, and stabilization of the obtained strain by the removal of the mutator allele by a second round of homologous recombination.

MATERIALS AND METHODS

Bacterial and Yeast Strains and Culture Conditions

The bacterial and yeast strains and plasmids used in this study are listed in Table 1. *H. polymorpha* DL1-LdU (*leu2* Δ *Aura3::lacZ*) and DL1-L (*leu2*) strains, derivatives of the DL1 (ATCC26012) strain [16, 19], were used as the parent strains to construct the mutator and subsequent mutant strains and as the control strain for phenotype comparison, respectively. *H. polymorpha* cells were grown in yeast peptone dextrose (YPD) medium (1% yeast extract, 2% bacto peptone, and 2% glucose). When necessary, YPM and YPX medium containing 2% methanol and 2% xylose, respectively, instead of glucose were used. For the selection of recombinant strains by auxotrophic markers, cells were incubated on a plate of synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.77 g/l drop-out supplement without uracil and/or leucine) at 37°C. When required, 0.5 mg/ml of 5-fluoroorotic acid (5-FOA) or 50 μ g/ml zeocin was added to the media for *H. polymorpha*. The *Escherichia coli* DH5 α strain was used for the construction of the vectors. The *E. coli* strain was cultured in Luria-Bertani (LB) medium supplemented with 100 μ g/ml of ampicillin or 25 μ g/ml of zeocin. Cell growth was measured by determining the

Table 1. Bacterial and yeast strains, and plasmids used in this study.

Strain or plasmid	Description	Source, or reference
<i>E. coli</i> strain		
DH5 α	F ⁻ Φ 80d <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>thi-1 gyrA96 relA1</i> Δ (<i>lacZYA-argF</i>)U169 λ ⁻	Laboratory stock
<i>H. polymorpha</i> strain		
DL1-L	Derivative of DL1 (ATCC 26012), <i>leu2</i>	[16]
DL1-LdU	Derivative of DL1, <i>leu2</i> Δ <i>Aura3::lacZ</i>	[19]
DL1-LC	DL1-LdU transformed with pHIF9, <i>URA3</i> ⁺ , <i>Zeo</i> ^R	This study
DL1-LM	DL1-LdU transformed with pHIF8, <i>URA3</i> ⁺ , <i>Zeo</i> ^R , <i>POL3*</i>	This study
Plasmid		
pUC18	Cloning vector, ColE1 <i>ori</i> , Amp ^R	Invitrogen
pUC-POL3N	pUC18 containing the 0.96 kb fragment encoding the N-terminal portion of HpPol3	This study
pUC-POL3NC	pUC18 containing the 2.9 kb fragment of HpPOL3* with its terminator sequence	This study
pUC-LEU2C	pUC18 containing the 0.56 kb C-terminus fragment of LEU2	This study
pUC-LEU2CN	pUC18 containing the 1.07 kb fragment of C-terminus and N-terminus of LEU2	This study
pHIMAZCH2	<i>H. polymorpha</i> expression vector	Unpublished
pHIF5	Derivative of pHIMAZCH2, <i>HpURA3</i> , <i>Zeo</i> ^R , pUC <i>ori</i>	This study
pHIF6	Derivative of pHIF5, <i>HpMOX</i> promoter	This study
pHIF7	Derivative of pHIF6, <i>HpPOL3*</i>	This study
pHIF8	Mutator plasmid, derivative of pHIF7, <i>LEU2CN</i>	This study
pHIF9	Derivative of pHIF8, control plasmid without <i>HpMOX</i> promoter and <i>HpPOL3*</i>	This study
pDLMOX	<i>H. polymorpha</i> - <i>E. coli</i> shuttle vector, <i>HpMOX</i> promoter, Amp ^R , HARS36, <i>LEU2</i>	[17]

optical density at 600 nm (OD₆₀₀) on a Shimadzu UV-2550 (Japan) spectrophotometer and by counting the colony-forming units per milliliter.

Construction of Mutator Plasmid

Standard recombinant DNA techniques were used to construct the vectors, as described previously [32]. The primer sequences used in this study are listed in Table 2. Restriction enzymes, DNA polymerase, and alkaline phosphatase were purchased from Takara Bio Inc. (Otsu, Japan). T4 DNA ligase and oligonucleotide primers (Table 2) were purchased from Elpisbio (Daejeon, Korea) and Bioneer Corp. (Daejeon, Korea), respectively. The sequences of the PCR-amplified DNA fragments were confirmed by DNA sequencing (Genotech Co. Ltd., Daejeon, Korea).

The mutator plasmid containing the *POL3** gene encoding proofreading-deficient DNA polymerase δ was constructed in multiple steps, as follows. First, the backbone pHIF5 vector containing pUCori, the zeocin-resistant gene (*Zeo*^R), and *HpURA3* was constructed by means of inverse polymerase chain reaction (iPCR) with the primers pHIF5-U and pHIF5-D, using the pHIMAZCH2 vector (unpublished data) as a template. Second, the 1.5 kb promoter region of the methanol oxidase (*MOX*) gene was PCR-amplified using the primers pMOX-N and pMOX-C with *H. polymorpha* DL1-L chromosomal DNA as the template. The amplified *MOX* promoter fragment was digested with *NheI* and *NdeI* and inserted into the *NheI*–*NdeI* digested pHIF5 vector, resulting in pHIF6. Third, the mutant *POL3* gene, in which the sequences encoding D³¹⁷I³¹⁸E³¹⁹ (GACATTGAG) were changed to encode A³¹⁷G³¹⁸A³¹⁹ (GCCGGCGCG), was prepared by PCR-based site-directed mutagenesis (Fig. 2B). The DNA fragment encoding the N-terminal portion of the mutated residues of Pol3 was PCR-amplified using the primers HpPOL3N-Fw and HpPOL3N-Rev with *H. polymorpha* DL1-L chromosomal DNA as the template. The 0.96 kb PCR product was digested with *EcoRI* and *SmaI* and ligated with the *EcoRI*–*SmaI*-digested pUC18 vector, yielding pUC-POL3N. The 2.9 kb DNA fragment encoding the C-terminal portion of the mutated residues of Pol3 and the terminator was amplified using the primers HpPOL3C-Fw and HpPOL3C-Rev, digested with *NgoMIV* and *HindIII*, and cloned into the *NgoMIV*–*HindIII*-digested pUC-POL3N, yielding pUC-POL3NC. The 3.9 kb *POL3** fragment was isolated after digestion of pUC-POL3NC with *NdeI* and *NsiI*

and cloned into *NdeI*–*PstI*-digested pHIF6, resulting in pHIF7. Fourth, the 0.56 kb DNA fragment encoding the C-terminal half of *Leu2* was PCR-amplified from the pDLMOX plasmid [17] using primers HpLEU2C-Fw and HpLEU2C-Rev. The PCR product was digested with *EcoRI*–*HindIII* and ligated with *EcoRI*–*HindIII*-digested pUC18, resulting in pUCLEU2C. The 0.52 kb DNA fragment encoding the N-terminal half of *Leu2* was PCR-amplified from the pDLMOX plasmid using primers HpLEU2N-Fw and HpLEU2N-Rev. The PCR-amplified N-terminal *LEU2* gene fragment was then digested with *NsiI* and *HindIII* and inserted into *NsiI*–*HindIII*-digested pUCLEU2C, resulting in pUC-LEU2CN. The 1.07 kb LEU2CN fragment was prepared from pUCLEU2CN by *PstI* digestion and ligated with the *NsiI*-digested and dephosphorylated pHIF7 vector, resulting in pHIF8 (Fig. 2A). To construct a control vector, pHIF8 was digested with *NheI* and *XbaI*, and the resulting 5,077 bp fragment with the *MOX* promoter and *POL3** sequence was self-ligated, resulting in pHIF9.

Chromosomal Integration and Removal of the Mutator Allele by Homologous Recombination

To construct the mutator strain, the pHIF8 plasmid was linearized by digestion with *NsiI* restriction enzyme, which cleaves between the LEU2C and LEU2N fragments, and was transformed into the *H. polymorpha* DL1-LdU strain [34] using the DMSO-lithium acetate (LiOAc) transformation method [12]. The transformants were screened on SC plates lacking uracil and containing zeocin. To confirm the chromosomal integration of the mutator allele, diagnostic PCR was performed with primers HpLEU2N-out-Fw and pMOX-C using the genomic DNA of the transformant as the template. The resulting strain was designated as the mutator strain *H. polymorpha* DL1-LM and was subjected to genome-wide random mutagenesis. After isolation of mutant strains by the action of the error-prone proofreading-deficient DNA polymerase δ , the mutator allele was removed from the chromosome, as described below. The *HpLEU2* fragment was PCR-amplified from the pDLMOX plasmid with primers HpLEU2N-Fw and HpLEU2C-Rev and transformed into a mutant strain generated from DL1-LM cells using the DMSO-lithium acetate method. Transformants growing on the YPD plates containing 0.5 mg/ml 5-FOA were selected and verified by diagnostic PCR to confirm the removal of the mutator allele. The control strain *H.*

Table 2. The primers used for this study.

Primer	Primer sequence (5' to 3') ^a
pHIF5-U	GAGCATATGCACGCTAGC <u>ACTGGCAATTTACCAGTAG</u> (<i>NdeI</i> , <i>NheI</i>)
pHIF5-D	CTCCATATGAACTGCAGTGACGTCGATCTCTACAACGA (<i>NdeI</i>)
pMOX-N	TTCGCTAGCCCTCGAGCTGTTTCGC (<i>NheI</i> , <i>XhoI</i>)
pMOX-C	GCGCATATGAATTCAGCCTTTTGT (<i>NdeI</i> , <i>EcoRI</i>)
HpPOL3N-Fw	TCTGAATTCGCTAGCCATATGAGTGCAACTACGTCG (<i>EcoRI</i> , <i>NheI</i> , <i>NdeI</i>)
HpPOL3N-Rev	TCTCCCGGGACGCGCCGGCAAATGACAAAATCCGCAA (<i>SmaI</i> , <i>NgoMIV</i>)
HpPOL3C-Fw	ATTTGCCGGCGCGTGTGCGGGCCGCAAGGGC (<i>NgoMIV</i>)
HpPOL3C-Rev	TGTAAGCTTATGCATCAGGTTGTCTAGGAGG (<i>HindIII</i> , <i>NsiI</i>)
HpLEU2N-Fw	TCTGAATTCATGCATTTGTGCTTCTCCCTG (<i>EcoRI</i> , <i>NsiI</i>)
HpLEU2N-Rev	CTCAAGCTTCGCTCTGCAGCGCCAA (<i>HindIII</i> , <i>PstI</i>)
HpLEU2C-Fw	CACGAATTCAGAGCGACCCACC (<i>EcoRI</i> , <i>PstI</i>)
HpLEU2C-Rev	TCTAAGCTTATGCATGAGTAGCTTGGCCACCT (<i>HindIII</i> , <i>NsiI</i>)
HpLEU2N-out-Fw	GTCAAACCTTAGCCACCTGTGC

^aUnderlined sequences indicate restriction sites for restriction enzymes shown in the parentheses.

polymorpha DL1-LC was constructed by integrating the linearized pHIF9 plasmid into the chromosome of the *H. polymorpha* DL1-LdU strain and screening on SC plates lacking uracil and containing zeocin.

Determination of Mutation Frequency

To compare the ability to generate mutations, the *H. polymorpha* DL1-L and DL1-LM strains, cultivated in a YPD liquid medium overnight at 37°C, were harvested and washed three times with 1 volume of 1× PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.2) by centrifugation at 13,000 rpm for 3 min. Cells were then re-inoculated in duplicate into YPM medium at the concentration of OD₆₀₀ = 0.3 and incubated at 37°C while shaking at 180 rpm. To induce the expression of the error-prone proofreading-deficient Pol3*, methanol corresponding to the final concentration of 2% (v/v) was added to the culture broth every 24 h. Samples were taken every 4 h and spread on SC plates with and without supplementation of 0.5 mg/ml 5-FOA [19] to count the frequency of the generation of *URA3* mutants from the *HpURA3*⁺ strain. The mutation frequency (number of mutant colonies/number of total colonies) in 1 ml of culture was calculated and graphed versus the culture time [45].

Screening and Stabilization of Mutant Strains with Enhanced Growth on Xylose

For the screening of the mutant strains that showed improved growth on the xylose medium, the *H. polymorpha* DL1-LM strain was grown in a 500-ml Erlenmeyer flask containing 50 ml of YPM medium for 7 days at 37°C while shaking at 180 rpm. Samples were taken every 12 h, washed with 1× PBS by centrifugation, and plated on YPX agar plates to isolate rapidly growing colonies. Selected colonies were inoculated in YPD medium and cultured overnight at 37°C while shaking at 180 rpm. Cell pellets harvested by centrifugation were resuspended in YPX and serially diluted to spot on YPX agar plates with *H. polymorpha* DL1 as a control strain to compare the growth phenotype. Enhanced growth of isolated mutant strains in comparison with the wild-type strain in the xylose medium was confirmed by measuring the cell growth in the YPX broth cultivating at 37°C with shaking at 180 rpm.

Determination of Ethanol Production

For the fermentation experiments, the *H. polymorpha* DL1-L, DL1-MXC-15, and DL1-MXC-15D strains were grown in a 250-ml Erlenmeyer flask containing 100 ml of a modified fermentation medium [0.67% yeast nitrogen base without amino acids, 0.05% yeast extract, 0.34% (NH₄)₂SO₄, and 0.02% leucine] supplemented with 10% glucose or xylose. Ethanol production by the *H. polymorpha* strains grown on xylose was compared with that of cells grown on glucose. Cultivation was performed at 37°C under respiro-fermentative conditions (shaking at 100 rpm) for 6 days. Ethanol was quantified using an EnzyChrom ethanol assay kit (ECET-100, BioAssay Systems) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Identification of the *H. polymorpha* *POL3* Gene

A close inspection of the whole-genome sequence of the *H. polymorpha* DL1 strain using the sequence of the DNA polymerase δ of the budding yeast *S. cerevisiae* with the

BLAST program revealed an open reading frame of 3,306 nucleotides encoding a polypeptide of 1,102 amino acids. The deduced amino acid sequence of HpPol3 showed strong amino acid sequence similarities ranging from 67.1% to 75.6% to Pol3 proteins of other yeast strains, such as *S. cerevisiae* (Sc), *Candida albicans* (Ca), and *Schizosaccharomyces pombe* (Sp) (Fig. 1). Furthermore, the amino acid sequence alignment revealed that HpPol3 contains all seven highly conserved DNA polymerase domains (Pol I to VII) [18, 29, 44], five exonuclease activity domains (Exo I to V) [37], and two C-terminal zinc finger domains (ZnF I and II) [33].

Development of a Mutator Strain Based on Proofreading-Deficient Pol3* and the Random Mutagenesis Strategy

It has been shown in *S. cerevisiae* and *S. pombe* that the proofreading-deficient DNA polymerase δ carrying a mutation at the conserved active site residues for 3'→5' exonuclease activity functions as a strong mutator that can successfully be used to isolate mutant strains with desired phenotypes, such as thermotolerance [3, 35]. Thus, we examined whether the random mutagenesis method based on the error-prone proofreading-deficient DNA polymerase δ may be applicable to the methylotrophic yeast *H. polymorpha*. Using PCR-based site-directed mutagenesis, we replaced the D³¹⁷E³¹⁹ residues in the 3'→5' exonuclease proofreading domain of the DNA polymerase δ of *H. polymorpha* with A³¹⁷G³¹⁹ to create an exonuclease-activity-deficient Pol3*. A mutator vector, pIHf8, containing the mutated DNA polymerase δ gene under the methanol inducible promoter of methanol oxidase *MOX* was constructed. This vector also contains the *URA3* gene and the *Zeo*^R gene as an auxotrophic marker and antibiotic zeocin-resistance marker for both *H. polymorpha* and *E. coli*, respectively. Because a stable episomal vector system is not available for *H. polymorpha*, heterologous DNA has to be integrated into the chromosome for stable maintenance. Thus, a linearized pHIF8 vector was transformed into the *H. polymorpha* DL1-LdU strain, and transformants with a chromosomally integrated mutator allele were selected by the zeocin-resistant phenotype and the disappearance of the uracil requirement for growth. After obtaining the desired mutant strains, the mutator allele was removed from the chromosome to avoid the accumulation of unwanted mutations by transforming the PCR-amplified DNA fragment containing *LEU2* sequence. The chromosomal integration and removal of the mutator allele were confirmed by PCR (data not shown).

Mutation Frequency of the *H. polymorpha* DL1-LM Mutator Strain

To determine the efficiency of genome-wide random mutagenesis using the proofreading-deficient DNA polymerase δ , *H. polymorpha* DL1-L wild-type, DL1-LC control, and



Fig. 1. Alignment of the amino acid sequences of HpPol3 with other yeast Pol3 proteins. Amino acid sequences of Pol3 proteins of the indicated yeasts (Hp = *H. polymorpha*, Ca = *Candida albicans* CAA21949.1 [40], Sc = *S. cerevisiae* CAA43922.1 [24], Sp = *Schizosaccharomyces pombe* CAA41968.1 [29]) were compared using ClustalW and Boxshade software (<http://www.ch.embnet.org>). Boxed sequences indicate the polymerase domains I to VII. Boxed sequences that are underlined indicate the exonuclease domains I to V. Underlined sequences indicate the C4-type zinc-finger domains I and II. The amino acid residues with asterisks in the exonuclease domain I indicate the conserved residues responsible for the 3' to 5' proofreading exonuclease activity.

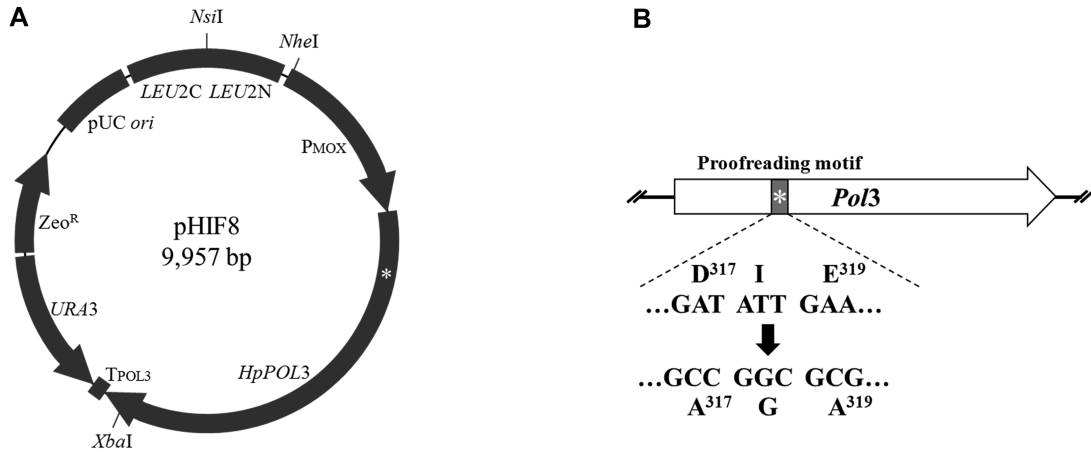


Fig. 2. Construction of the pHIF8 mutator plasmid. (A) The mutator plasmid pHIF8 contains the mutant *POL3** gene encoding the proofreading-deficient mutant DNA polymerase δ (HpPol3*), the promoter of methanol oxidase (*PMOX*), an auxotrophic marker *URA3*, the antibiotic zeocin-resistant gene (*Zeo^R*), the replicate origin of pUC (*pUC ori*), and the C-terminal and N-terminal fragments of the *LEU2* gene. (B) To generate the proofreading-deficient mutant DNA polymerase δ , sequences encoding amino acid residues D³¹⁷ I E³¹⁹ in the 3' to 5' exonuclease proofreading domain of *H. polymorpha* Pol3 were altered by PCR-based site-directed mutagenesis to A³¹⁷ G A³¹⁹.

DL1-LM mutator strains were cultured in YPM medium at 37°C while shaking at 180 rpm and supplementing methanol at 2% every 24 h. Samples were taken every 12 h and spread on SC plates and SC plates containing 0.5 mg/ml 5-FOA to count the frequency of the generation of a *URA3*

mutant strain resistant to the pyrimidine analog 5-FOA [5]. The *H. polymorpha* DL1-LM mutator strain produced *URA3* mutant colonies at a frequency of $9.2 (0.5) \times 10^{-7}$ after being grown in YPM for 144 h. The mutation frequency of the mutator strain was about 50- to 90-fold

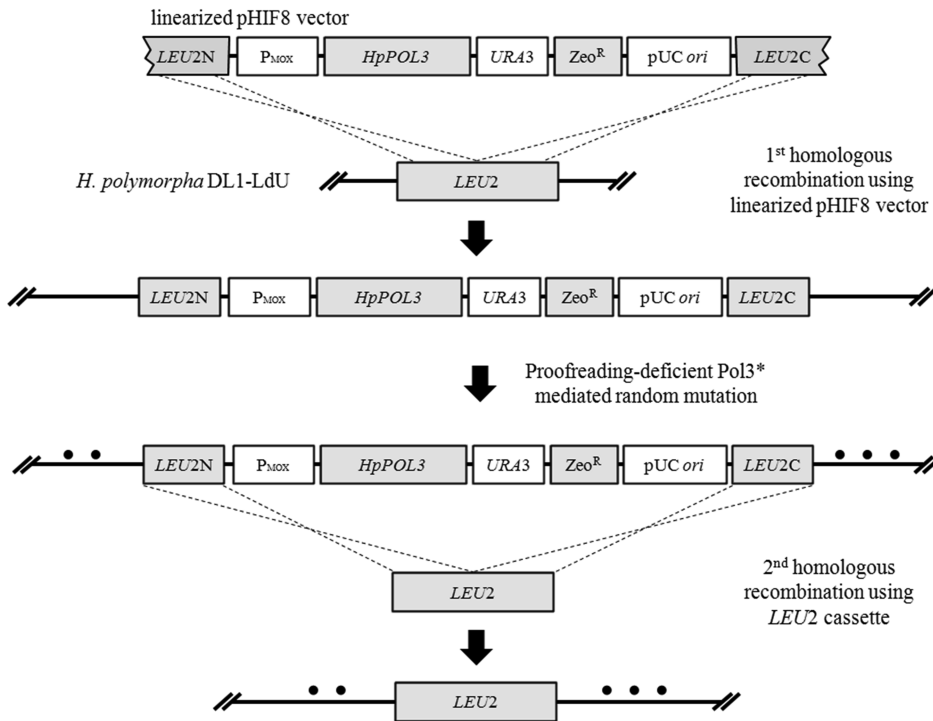


Fig. 3. Schematic strategy for genome-wide mutagenesis using the error-prone proofreading-deficient DNA polymerase δ . The mutator plasmid pHIF8 was linearized and integrated into the chromosome of the *H. polymorpha* DL1-LdU strain by homologous recombination. The resulting mutator strain *H. polymorpha* DL1-LM was subjected to random mutagenesis by the action of the dominant negative expression of the error-prone proofreading-deficient DNA polymerase δ under control of the *MOX* promoter. After the isolation of the mutant strain with the desired phenotype, the mutator allele was removed by a second round of homologous recombination to stabilize the mutant, avoiding the accumulation of additional mutations.

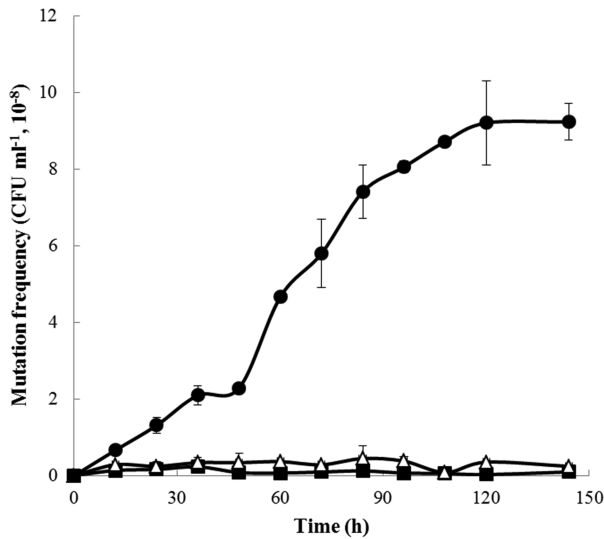


Fig. 4. Mutation frequency of the mutator strain.

The *H. polymorpha* DL1-L wild-type (closed squares), DL1-LC control (open triangles), and DL1-LM mutator (closed circles) strains were cultured in YPM medium to induce the expression of the error-prone proofreading-deficient DNA polymerase δ , and samples were taken at indicated time intervals and spread on YPD and YPD plates containing 5-FOA. The mutation frequencies were calculated based on the number of colonies grown on both plates after 60 h of incubation at 37°C. To induce the expression of the Pol3*, 2.0% methanol was added at 24, 48, 72, 96, and 120 h of cultivation.

higher than that of the DL1-L wild-type and DL1-LC control strains (Fig. 4). It is noteworthy that an increase of approximately 130-fold in mutation frequency was observed in the *S. cerevisiae* mutator strain harboring the plasmid containing the error-prone DNA polymerase δ gene, compared with the strain harboring the empty plasmid [25, 36].

Isolation of *H. polymorpha* Mutant Strains with Enhanced Xylose Metabolism

To assess the possibility to employing the error-prone proofreading-deficient DNA polymerase for the generation of a mutant strain with a phenotype that may involve mutations at multiple unidentified genes, we isolated mutant strains showing improved growth on xylose, a pentose sugar abundant in lignocellulosic biomass (Fig. 5A). One of the isolated mutant strains, *H. polymorpha* DL1-MXC-15, showed an increased growth rate compared with the wild-type strain when cultured in YPX medium. In addition, its final cell density after 32 h of incubation was about 74% higher than that of the wild-type strain (Fig. 5B). Furthermore, it was found that the enhanced growth phenotype of the mutant strain was maintained even after the removal of the mutator allele by homologous recombination to prevent the accumulation of additional mutation (Fig. 5B). These results indicate that the mutant phenotype was acquired by random mutation mediated by the error-prone proofreading-

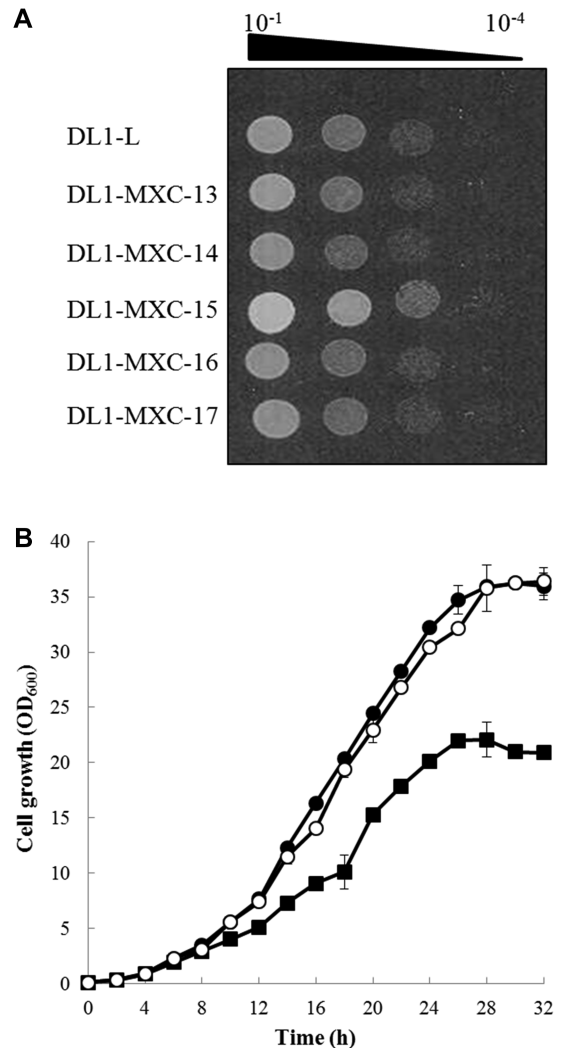


Fig. 5. Isolation of mutant strains with enhanced xylose metabolism. (A) Aliquots of mutator strain cultured in YPM medium were spread on YPX plates containing 2% xylose, and rapidly growing colonies were selected for further characterization by spot analyses on YPX plates. (B) Growth of the wild-type (DL1-L, closed squares) mutant strain with enhanced xylose metabolism (DL1-MXC-15, closed circles) and its derivative stabilized by the removal of the mutator allele (DL1-MXC-15D, open circles) in YPX medium.

deficient DNA polymerase δ , but not by the integration of the mutator allele itself. Because *H. polymorpha* is known to be able to ferment xylose, we examined the ability of the above mutant strains to produce ethanol from xylose. However, we were not able to observe any significant change in ethanol production in mutant strains compared with that of the wild-type strain (data not shown). This result suggests that to obtain a mutant *H. polymorpha* strain with enhanced ability to ferment xylose, an additional round of mutagenesis should be performed in combination with a proper screening strategy. For example, it may be possible to select such a mutant on a xylose medium based on the

ability to support the growth of an indicator strain that can grow on ethanol, as previously reported [11]. It will also be possible to construct a reporter strain using the recently identified ethanol-inducible promoter of the *HpADH3* gene of *H. polymorpha* [39].

Recently, it has been reported based on a genome-wide analysis of *S. cerevisiae* using high-throughput next generation sequencing that mutator mutagenesis using the error-prone proofreading-deficient DNA polymerase δ showed about a seven times higher mutation frequency than chemical mutagenesis using ethyl methanesulfonate (EMS) [20]. Furthermore, it has been demonstrated that in contrast to the EMS method, which mainly produces G/C to A/T transitions, the mutator method mainly produces transversions, resulting in more diverse amino acid substitution patterns [36]. A proofreading-deficient DNA polymerase-based random mutagenesis strategy has been successfully applied as a mutagenesis technique to generate novel strains with desired traits in various microorganisms, such as *S. cerevisiae* [1-3, 35], *S. pombe* [3], *Ashbya gossypii* [27], *E. coli* [41], and *Bradyrhizobium japonicum* [14]. However, this has yet to be applied to a methylotrophic type of yeast. In this study, we identified the DNA polymerase δ of *H. polymorpha* and developed an efficient *in vivo* genome-wide random mutagenesis strategy. The mutator system was designed to be readily introduced into the genome of the target strain and then removed after the generation of the desired mutation, showing a higher mutation frequency by more than 50-fold as compared with the wild-type strain. Taken together, our results suggest that the mutator system will be useful for the genome-wide engineering of *H. polymorpha* to obtain mutant strains, especially those related to unknown or multiple genes on the chromosome.

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